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14. ABSTRACT

This proposal is to apply genome-wide technologies to analyze ovarian cancer genome and transcriptome in parallel in the same ovarian tumors to reveal genes with concurrent genomic amplification and transcription upregulation. We propose to use digital karyotyping as the discovery tool to identify genomic amplification in ovarian carcinomas, and based on the results from 7 digital karyotyping libraries, we are able to identify several novel amplifications with high frequency as well as known amplified oncogenes including Cyclin E1, AKT2, LMyc. In the past year, we have focused on a novel amplicon located at chromosome 11q since it is frequently amplified in high-grade ovarian serous carcinomas. Combined genome and transcriptome analysis was performed at 48 tumors, and the results indicate Rsf-1 as the gene with most consistent DNA amplification as well as transcript and protein up-regulation. Furthermore, FISH analysis on a panel of ~110 ovarian carcinomas showed that patients with Rsf-1 amplification had significantly shorter overall survival that those without. The function of Rsf-1 in proliferation was also established by RNAi knock-down assays. Currently we are focusing on Rsf-1 and other candidate oncogenes identified by digital karyotyping to reveal their clinical and biological significance in ovarian carcinomas.

15. SUBJECT TERMS
amplification, genetics, carcinoma

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Introduction

Identification of new oncogenes that participate in the development of ovarian carcinoma holds great promise to develop new strategies for diagnosing and treating this devastating disease. Several elegant studies have employed gene expression profiling as the discovery tool (such as DNA-based microarray) and have identified a myriad of candidate markers associated with ovarian carcinomas. Several of these over-expressed genes have proven to be novel biomarkers of cancer [1]. However, it is challenging to use such approach alone to distinguish the truly important genes that directly drive tumor progression from a larger number of the “passenger” genes that are co-overexpressed but lack the biological roles in tumor development. This is because gene expression is dynamic, depending on both genetic program and tumor microenvironment. In contrast, molecular genetic changes such as gene amplification and point mutation are inheritable traits as a result of clonal selection and they likely confer a growth advantage to tumor cells and propel tumorigenesis.

We hypothesize that a comprehensive analysis of ovarian cancer genome by combining genomic analysis (amplification and somatic mutation) and gene expression analysis would significantly facilitate the identification of oncogenes that directly contribute to the development of ovarian tumors. The main objective is to identify the oncogenic alterations that participate in the development of ovarian serous carcinoma, the most common and malignant type of ovarian cancer. This goal will be achieved through a parallel analysis of cancer genome and transcriptome. This approach will identify genes demonstrating increases in copy numbers of both genomic DNA and mRNA and those genes will be characterized through mutational analysis. Digital karyotyping, a technology developed in our research team that permits an analysis of cancer genome at high resolution [2], was employed to scan ovarian cancer genome. SAGE (serial analysis of gene expression) [3] and high throughput quantitative real-time PCR were performed to reveal the transcriptome in each amplified region detected by digital karyotyping. Using this approach, we have identified several candidate oncogenes with concurrent gene amplification and transcript/protein up-regulation and currently, we are focusing on their functional significances in tumorigenesis.

Body

There are no substantial changes or modifications of the original statements. The accomplishments associated with each task outlined in the approved statement of work are detailed, point by point in the followings:

Task 1. To identify genome-wide amplifications in 8 ovarian serous carcinomas using Digital Karyotyping.

In the 1st year funding period, we have exceeded our initial goal and finish digital karyotyping libraries for 7 serous carcinomas. Novel discrete gene amplifications were identified and parts of the data were published in PNAS and current review of Oncology [4] [5]. We have focused on a frequently amplified region on chromosome 11q13.2 since this amplicon was detected in 3 out of 7 digital karyotyping libraries. The high resolution readouts of digital karyotyping has facilitated the amplicon mapping and narrowed down the region to only 1.8Mb. Large-scale FISH analysis on clinical specimen demonstrated that amplification of 11q13.2 was associated with a worse disease outcome in patients with serous type of ovarian carcinomas.

In addition to the novel amplicons identified, digital karyotyping demonstrated frequent amplification of cyclin E1 and Akt2, and to a lesser extent for L-Myc. These results will pave the ways for future directions for development of target-based therapies and prognosis markers.

Task 2. To analyze genome-wide mRNA expression in the same tumors studied in AIM 1.

It is expected that each amplicon identified in Aim 1 harbors only one or few oncogenes(s) among hundreds of co-amplified "passenger" genes that are not involved in tumorigenesis. In order to distinguish the "driver" genes from the "passenger" genes and thus narrow down the candidate oncogenes list, we proposed to correlate the gene amplification and expression in the same tumors. The rationale is that oncogenes that are amplified are always over-expressed at mRNA and protein levels. In contrast, the co-amplified "passenger" genes that are unrelated to tumor development are less likely to be over-expressed [6]. We performed bioinformatics to search for all annotated genes in each amplicon, used Primer 3 program to design PCR primers, and performed quantitative real-time PCR for all the genes within each amplicon for the tumors with specific amplification. The result is further analyzed in Task 3.

Task 3. To generate transcriptome map and compare to genomic map to identify the genes with both amplification and over-expression in the same tumor samples.

The data derived from Task 2 was used to generate a weather map and transcriptome results from amplified tumors were compared with non-amplified tumors. Wilcoson test was performed to compute the difference between these two groups. Genes with most statistically difference was prioritized for further analysis. Using this approach, we successfully analyzed the chr11q13.2 amplicon and identified a potential driver gene, Rsf-1, with most significant correlation of gene over-expression and amplification.

Task 4. To identify somatic mutations in candidate genes with both amplification and over-expression.

We have performed mutational analysis for the candidate genes with the chr11q13.2 amplicon including Rsf-1, PAK4 and Gab2 in a panel of purified ovarian carcinomas. However so far, no somatic mutation was identified indicating it is a rare event comparing to gene amplification.

Key Research Accomplishments

- Generate 7 digital karyotyping libraries and identify novel somatic amplifications including the one located on chromosome 11q13.2

- Generate the transcript expression profiles of 11q13.2 amplicon in a panel of tumors with and without 11q13.2 amplification.

- Statistically analyze the transcript expression profiles and identified gene(s) with most consistent up-regulation in tumors with amplification. Rsf-1 was identified as the most promising gene in 11q13.2

- Functional knock-down Rsf-1 and other candidate oncogene in line to establish their oncogenic roles.

- Perform large scale FISH analysis on ovarian carcinoma specimens and establish Rsf-1 amplification as novel prognosis marker.

Reportable Outcomes

Articles published in the 1st year of funding period (January, 2005-December 2005)

- I-M Shih, J J-C Sheu, A Santillan, K Nakayama, M J Yen, R E. Bristow, R Vang, G Parmigiani, RJ Kurman, CG Trope, B Davidson and **T-L Wang** (2005) Amplification of a Chromatin Remodeling Gene, Rsf-1/HBXAP, in Ovarian Carcinoma. ***Proc Natl Acad Sci USA*** 99: 3076-3080.
- IM Shih and **T-L Wang** (2005) Exploring cancer genome using innovative technologies. ***Curr Opin Oncol***, 17:33-38.
- G Singer, R Stohr, L Cope, R Dehari, A Hartmann, D-F Cao, **T-L Wang**, RJ Kurman, IM Shih (2005) Patterns of p53 mutations separate ovarian serous borderline tumors, low and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis. ***Am J Surg Pathol*** 29:218-224, 2005.
- Y-C Chen, G Pohl, **T-L Wang**, PJ Morin, B Risberg, GB Christesen, A Yu, B Davidson, IM Shih (2005) Apolipoprotein E is required for cell proliferation and survival in ovarian cancer. ***Cancer Research***, 65:331-337.
- G Pohl, C-L Ho, RJ Kurman, R Bristow, **T-L Wang**, IM Shih (2005) Inactivation of the MAPK pathway as a potential target-based therapy in ovarian serous tumors with KRAS or BRAF mutations. ***Cancer Research***, 65:1994-2000.
- DW Parsons*, **T-L Wang***, Y Samuels, A Bardelli, J Cummins, L DeLong, N Silliman, J Ptak, S Szabo, K W Kinzler, C Lengauer, B Vogelstein and VE Velculescu‡ (2005) Mutational analysis of the serine/threonine kinome in colorectal cancers identifies alterations in PI3K pathway genes. ***Nature***, 436:792

Articles submitted for review

- J T. Park, M Li, N Nakayama, B Davidson, Z Zheng, R Kurman, C Eberhart1, IM Shih, **T-L Wang**. Notch3 gene amplification in ovarian cancer. ***Cancer Research*** (in revision)
- K Nakayama, N Nakayama, B Davidson, R J Kurman, I-M Shih, **T-L Wang**. Deletion of MKK4 in high-grade ovarian serous carcinomas. (*submitted*)

Research resource

A centralized web deposit of digital karyotyping data is initiated by Cancer Genome Anatomy Project, NCI (http://cgap-stage.nci.nih.gov/SAGE/DKViewHome). All the sequence tags from each digital karyotyping libraries can be retrieved from this website and the browser provides bioinformatics tools to analyze the DNA copy number alterations using varying parameters, including window scales, and size and fold of alterations. Currently there have been 22 libraries deposited and in the future all the data generated from this project will be publicly available at this web link.

Trainees who received awards using this funding resource

Jim Sheu, Ph.D. Research Fellow 1st Place in Basic Science Award, Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, April 28, 2005 (Advisor: Tian-Li Wang)

Conclusions

Ovarian epithelial tumor is the most common type most lethal type of gynecologic malignancy. The main purpose of this project is to delineate the genomic amplification in ovarian serous tumors and identity the genes that contribute to tumor progression. The 1st year of DoD project has made progress toward this goal as we have accomplished many of the tasks proposed in the timetable. Using digital karyotyping, we have identified a host of novel genomic alterations in ovarian carcinomas. We have performed detailed transciptome analysis of 11q13 amplicon and identified Rsf-1 as the gene with most consistent gene amplification and transcript/protein up-regulation. Furthermore, survival data showed that the patients with Rsf-1 amplification fared worse than patients without the ramped-up genes. The function of Rsf-1 in proliferation was also established by RNAi knock-down assays.

Implications and significance of the accomplished research findings: This proposal represents the study to use advanced genome-wide technologies to search the culprit oncogenes in ovarian cancer. Our research team has developed Digital Karyotyping to permit a systematical analysis of cancer genome at high resolution. This project is proposed to perform Digital Karyotyping to search the abnormal amplifications (many copies of the chromosomal elements in cancer but not in the normal ovaries). It has been well known that the amplified regions usually harbor over-expressed oncogenes that drive cancer development. Therefore, we propose to systematically analyze transcription level of all the genes within those amplified regions to narrow down the candidate oncogenes list. Using this approach, we have identified several candidate oncogenes in novel amplicons and have published one of them in 2005. We will focus on those candidate oncogenes for further studies to reveal their clinical and biological significance in ovarian carcinomas.

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Appendices

Representative Publications in the 1st year of project:

1. M Shih and T-L Wang (2005) Exploring cancer genome using innovative technologies. Curr Opin Oncol, 17:33-38.
2. I-M Shih, J J-C Sheu, A Santillan, K Nakayama, M J Yen, R E. Bristow, R Vang, G Parmigiani, RJ Kurman, CG Trope, B Davidson and T-L Wang (2005) Amplification of a Chromatin Remodeling Gene, Rsf-1/HBXAP, in Ovarian Carcinoma. Proc Natl Acad Sci USA 99: 3076-3080.

Apply innovative technologies to explore cancer genome

Ie-Ming Shih and Tian-Li Wang

Purpose of review
Molecular genetic alterations characterize the development of human cancer. Recent advances in molecular genetic technology and the success of the human genome project have empowered investigators with new tools in dissecting the cancer genome for discovery of new cancer-associated genes. The purpose of this review is to highlight the emerging molecular genetic methodologies and summarize their principles, applications, and potential technical challenges. The critical issue in sample preparation and a strategy that combines different molecular techniques to facilitate the identification of novel cancer-associated genes will be discussed.

Recent findings
Digital karyotyping and array-based techniques including array comparative genomic hybridization and representational oligonucleotide microarray analysis have been recently developed to study the genomic landscape in human cancer. These innovations provide tools to quantitatively measure DNA copy number changes in cancer and to map those changes directly onto the human genome. Digital karyotyping is based on counting the sequence tags that are distributed in the human genome and thus, it provides a digital readout to precisely outline the amplified and deleted chromosomal regions. Array-based technologies, on the other hand, compare the content of cancer and reference genomes followed by localizing the amplified or deleted signals in chromosomal regions using an array hybridization technique. In addition, a high-throughput mutational analysis platform has been available for a large-scale mutational analysis by using an automated capillary sequencing device and sophisticated bioinformatic tools. A number of examples have demonstrated the promise of these new molecular genetic approaches in identifying several potential new oncogenes and tumor suppressors.

Summary
As compared with conventional cytogenetics methods, digital karyotyping, array comparative genomic hybridization, and representational oligonucleotide microarray analysis provide an unprecedented mapping resolution that allows a precise localization of the amplified and deleted chromosomal regions. These technologies can be combined with gene expression profiling and high-throughput mutational analysis to facilitate the search for new cancer-associated genes. It is expected that applying these new technologies will lead to discovery of a host of novel oncogenes and tumor suppressors, which will have a significant impact in our understanding of tumorigenesis and in the clinical management of cancer patients.

Keywords
cancer genome, amplification, deletion, mutation, digital karyotyping, array comparative genomic hybridization, representational oligonucleotide microarray analysis

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Current Opinion in Oncology 2005, 17:33–38

Abbreviations

CGH	comparative genomic hybridization
ROMA	representational oligonucleotide microarray analysis

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Introduction

It is well known that tumors develop as a result of accumulated molecular genetic or genomic alterations including amplification, deletion, point mutation, and translocation [1]. Analysis of molecular genetic changes has historically led to identification of oncogenes and tumor suppressors. For example, characterization of amplified regions of the breast cancer genome has revealed several important oncogenes including Her-2/neu and c-Myc. Studies of these alterations are critical to understanding the molecular basis of cancer and providing potential diagnostic/outcome markers and therapeutic targets for cancer patients [1,2]. For example, amplification of cyclin E and Her2/neu is frequently associated with advanced stages of disease and a poor clinical outcome in ovarian cancer patients [3–5]. Antagonizing the oncogenic function using the anti-Her2/neu antibody therapy (trastuzumab; Herceptin, Genentech, CA, USA) prolongs the disease-free interval in patients

with Her2/neu gene amplification [6]. Although several oncogenes and tumor suppressors have been studied in past decades, many oncogenes and tumor suppressors remain to be identified.

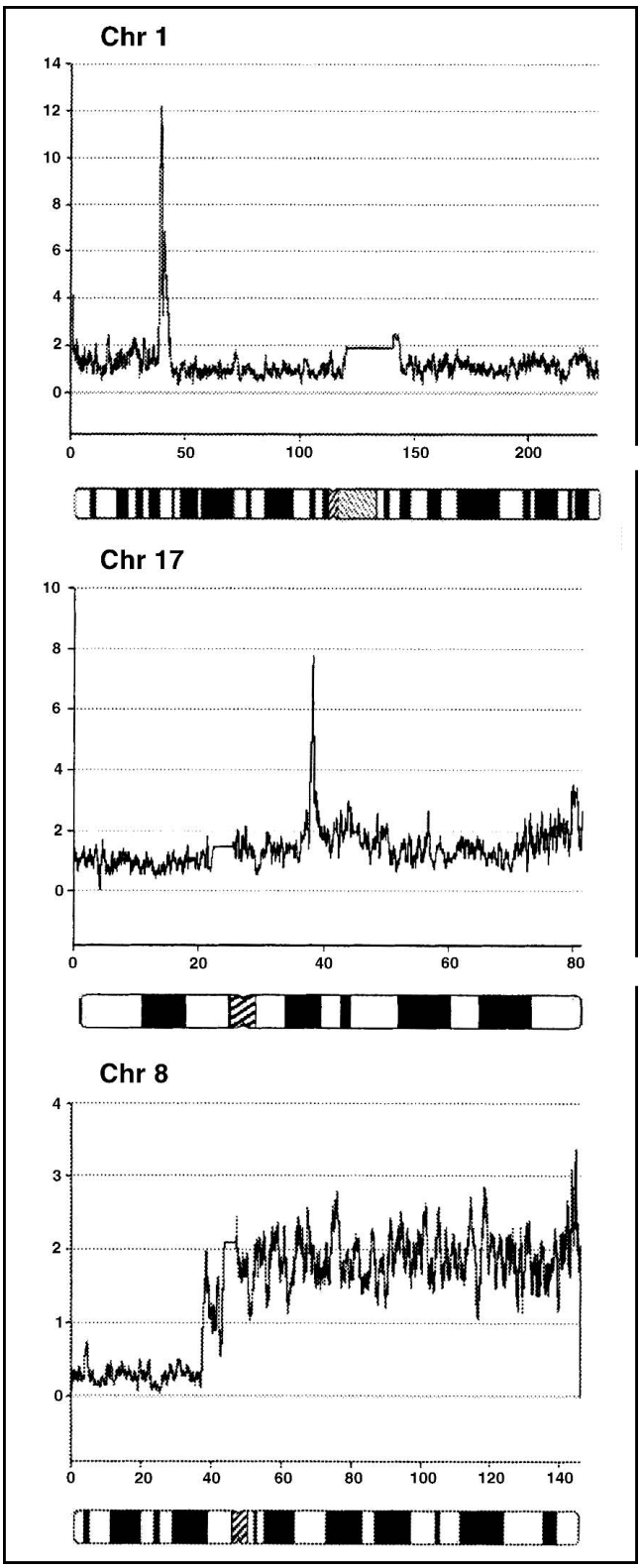
The rate-limiting step in discovering new oncogenes and tumor suppressors has been the lack of effective approaches for their discovery. Several elegant studies have used gene expression profiling as the discovery tool and have identified myriad candidate markers associated with different types of cancer [7,8]. However, the challenge is how to use such an expression-based approach alone to distinguish the cancer-driving genes that directly propel tumor progression from a larger number of passenger genes that are concurrently overexpressed but lack biologic relevance in tumor development. This is because gene expression is dynamic, depending on both genetic and epigenetic programs in tumor cells. In contrast, molecular genetic changes, such as alterations in DNA copy number (for example, amplifications and deletions), and point mutations are inheritable traits and are the result of Darwinian selection in tumors because of growth advantage conferred by these alterations [1]. Conventional methods used to reveal DNA copy number changes include comparative genomic hybridization (CGH), representational difference analysis, spectral karyotyping/metaphase fluorescence *in situ* hybridization, and conventional cytogenetics. These methods have aided in the identification of genetic aberrations in human cancer, but they generally have a limited mapping resolution (5 to 20 Mb) and, therefore, are not suitable to detect smaller chromosomal alterations. The success of the human genome database has accelerated cancer genome study because it provides precise and detailed maps to facilitate chromosomal mapping and localization of potential oncogenes and tumor suppressors. However, the question remains regarding the availability of suitable technical platforms that would allow a comprehensive survey of the cancer genome. It cannot be overemphasized that a high-resolution readout of the techniques is essential because precise genomic locations of amplification and deletion are required for further identification of novel oncogenes and tumor suppressors.

Recent developments of technologies including digital karyotyping, array-based CGH, and representational oligonucleotide microarray analysis (ROMA) provide molecular solutions, for the first time, to detect DNA copy number changes at a genome-wide scale with an excellent resolution. In addition, an automated capillary sequencing platform has become available for a large-scale mutational analysis. In this article, we will focus on reviewing these new technical advances and will briefly discuss a critical issue in sample preparation. Finally, we will review a rational strategy by combining genomic analysis, gene expression profiling, and large-scale mutational analysis to expedite the identification of oncogenes and tumor suppressors.

Digital karyotyping

Digital karyotyping has recently been developed for a genome-wide analysis of DNA copy number alterations at high resolution [9]. The principle of this approach is similar to the serial analysis of gene expression method [8,10], which is based on the isolation and enumeration of short sequence tags. However, the sequence tags in digital karyotyping are obtained from genomic DNA rather than from mRNA, and they are isolated by different methods. These tags (21 bp each) contain sufficient information that allows assigning the tag sequences to their corresponding genomic loci from which they are derived. After isolation, the tags are ligated to each other and are cloned into bacteria. Therefore, every bacterial clone represents homogeneous plasmid that contains a certain number of different tags (approximately 32 tags). Generally, approximately 5000 clones are sequenced from each tumor sample to establish a digital karyotyping library that collects a total of 160,000 tags (32 × 5000). Populations of tags can then be uniquely matched to the assembled genomic sequence in a public domain, allowing observed tags to be sequentially ordered along each chromosome. The number of each unique tag along each chromosome can be used to quantitatively evaluate DNA content in tumor samples. To prove the above concept, digital karyotyping libraries have been generated from a colorectal cancer cell line (DiFi) and an ovarian cell line (SKOV3) in which the preexisting CGH data are available for comparison. Digital karyotyping identified all the known chromosomal alterations including whole chromosome changes, gains or losses of chromosomal arms, and interstitial amplifications or deletions in both cell lines. More importantly, digital karyotyping revealed several distinct genetic alterations including amplifications of relatively small amplicons (less than 1 Mb) and homozygous deletions that have never been previously described using other methods. For example, the SKOV3 cell line was known to contain Her2/neu gene amplification [11]. Digital karyotyping of SKOV3 cells is able to demonstrate a distinct amplification in the Her2/neu locus (17q12) (Fig. 1B), which was not evident by CGH or SKY analysis [12,13]. These analyses suggest that a potentially large number of undiscovered copy number alterations exist in cancer genomes and many of these could be detected through digital karyotyping. Examples of amplification and deletion revealed by digital karyotyping are shown in Figure 1. Digital karyotyping has been recently applied in identifying specific gene amplification that is associated with resistance to chemotherapy [14••]. In that study, a significant fraction of colorectal cancer patients undergoing 5-fluorouracil treatment were found to have amplification of the thy-

Figure 1. Detection of amplified and deleted chromosomal regions by digital karyotyping



Digital karyotyping libraries were generated using isolated tumor cells from ovarian serous carcinoma tissues. Analysis of the libraries reveals two distinct amplifications in chromosomes 1p34.2 and 17q12, which are know to contain L-myc and Her2/neu oncogenes, respectively (top and center). A deletion in chromosome 8p is also evident (bottom). The chromosome ideograms are aligned with the results of digital karyotyping for reference.

midylate synthase (TYMS) locus. Patients with TYMS gene amplification have been shown to have a shorter survival than those do not. This finding could have significant implications for the clinical management of cancer patients with colorectal cancer.

The major advantage of digital karyotyping in exploring the cancer genome is its higher resolution than the conventional methods. This is because the technique involves using SacI as the mapping enzyme in which the enzyme sites are abundant in human genome (spanning an interval of approximately 4 kb). Therefore, a high resolution can be theoretically achieved if a sufficient number of tags can be obtained (sequenced). Furthermore, digital karyotyping provides unbiased gene dosage readout because digital karyotyping directly counts the tags in contrast to the analog signal generated by hybridization that is associated with an array format. However, it is possible that a small portion of the genome has a lower density of mapping enzyme SacI sites and could be incompletely evaluated by digital karyotyping. This potential problem could be overcome through the application of different mapping and fragmenting enzymes.

Array-based technologies to detect DNA copy number changes: array comparative genomic hybridization and representational oligonucleotide microarray analysis

In contrast to a tag counting strategy used in digital karyotyping, two related technologies, array CGH and ROMA, have been developed by using the format of DNA microarrays to map the loci of amplification and deletion. Array-based CGH is a technique that combines conventional CGH and DNA microarray for detection of DNA copy number changes [15–17]. Conventional CGH has been widely used in identifying chromosomal imbalances in cancers, but the relatively low mapping resolution (5 to 20 Mb) has limited its use as a discovery tool for novel cancer-associated genes [18]. In contrast, array CGH takes the advantages of CGH but it analyzes genomic alterations using DNA microarrays instead of metaphase chromosomes. In this way, the assay resolution increases as thousands of genomic targets representing different genomic locations can be analyzed simultaneously. In array CGH, total genomic DNA from a tumor and a normal cell population are labeled with different fluorochromes and hybridized to arrayed genomic components of cDNA [16] or large genomic fragments such as bacteria artificial chromosomes and phage artificial chromosome [15]. The ratio of the fluorescence intensities on each spot in the array is proportionally correlated to the copy number of the corresponding sequences in the tumor. Comparison of ratios on overlapping clones should allow amplifications and deletions in DNA copy number to be mapped in the genome. Thus, array CGH is a technique by which variation in relative copy num-

bers between two genomes can be analyzed by competitive hybridization to DNA microarrays [19–21]. Several recent studies have shown the potential of array CGH in detecting DNA copy number alterations in fallopian tube carcinomas [22], oral squamous carcinoma [23], bladder cancer [24], pancreatic cancer [25], chronic lymphocytic leukemia [26], and gastric cancer [27,28]. Although CGH array can provide a number of advantages over the conventional cytogenetics approaches including higher resolution and throughput, it has been previously limited by the availability of genomic clones that can be spotted as targets. Recent attempts, however, have improved the resolution of array CGH to approximately 1 Mb by establishing a high-density array [29••,30]. Furthermore, new analysis tools have been recently developed to provide a more uniform and convenient analysis platform for array CGH [31].

Representational oligonucleotide microarray analysis is another array-based technique for the detection of genome copy number variation. The method is modified from the representational difference analysis [32] by utilizing the microarray format [33••]. The principle of ROMA is based on the concept of genomic representation that is generated by amplifying restriction enzyme (such as Bgl II) digested genomic fragments from samples. The representative genomic fragments hybridize to the oligonucleotides in arrays that are designed from the human genome sequence assembly. The major advantage of using a representation strategy is to minimize the genome complexity and therefore maximize the signal-to-background ratio. Using ROMA, investigators are able to detect regions of copy number variations between cancer and normal genomes and between normal human genomes [33••,34]. Currently, ROMA can reach a resolution up to 30 to 35 kb, and further refinement of ROMA will promise to reveal an even better resolution by designing a higher density array.

High-throughput mutational analysis

Besides DNA copy number changes, somatic point mutation represents another salient feature of molecular genetic changes in cancer because the point mutation can lead to activation of oncogenes and inactivation of tumor suppressor genes. The discovery of somatic point mutations, in the past, has been challenged by an unsatisfied throughput inherent to the traditional DNA sequencing and heteroduplex analysis. Recently, with the advance of human genome assembly, a high-throughput sequencing pipeline is made possible by the application of capillary sequencers and the availability of bioinformatic software. For example, the current capacity of a single capillary nucleotide sequencer with a 384-well format can analyze at least 2304 sequencing reactions per day (384 reactions/plate × 6 plates/d). This high-throughput platform permits a systemic scan of cancer genome at the nucleotide level in a short time [35]. This format has been successfully used to screen protein families that could contain genes critical in tumor development [36–38••].

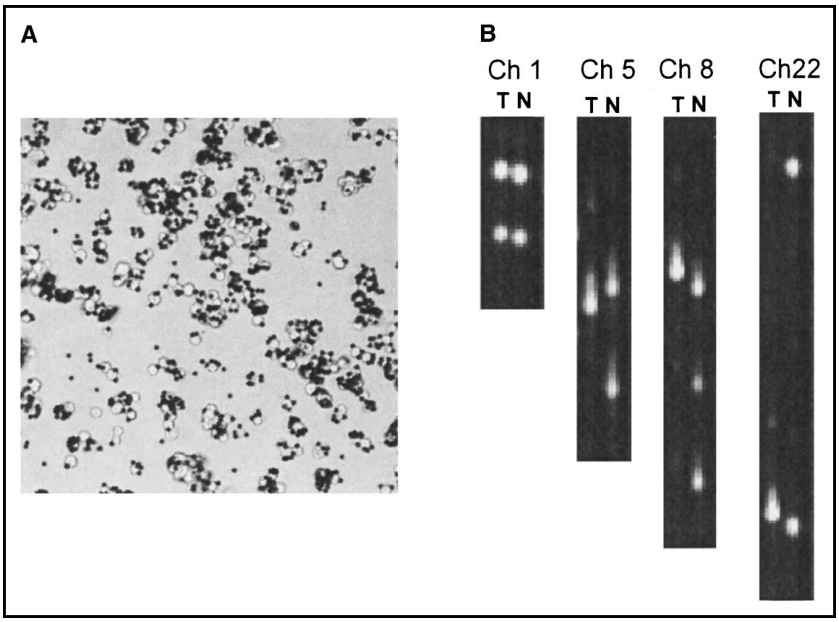
Identification of the BRAF oncogene is another example of the power of high-throughput mutation analysis in finding new oncogenes. Davies *et al.* [39] used the heteroduplex method and high-throughput capillary sequencers to screen mutations in genes belonging to the RAS-RAF-MEK-ERK (MAP) kinase pathway and found that activating mutations in BRAF occurred in 66% of melanomas. Functional study demonstrates that mutated BRAF proteins have elevated kinase activity and are able to transform NIH3T3 cells. Subsequent studies have further shown frequent BRAF mutations in other specific types of cancer, including papillary thyroid carcinomas (69%) [40] and low-grade ovarian serous tumors (30%) [41•]. Because activating mutations within the protein kinase family may be amenable to therapeutic intervention such as kinase inhibitors, these studies suggest a potential target-based therapy for those patients whose tumors harbor BRAF mutations.

Critical issues in preparation of tumor DNA

As new molecular genetic technologies are emerging, isolation of tumor DNA has become a critical issue because all the above mentioned techniques will not be useful unless the tumor DNA is enriched. Detection of homozygous deletions and point mutations would be equivocal and the folds of amplification obscure in the presence of substantial amounts of contaminated DNA from normal tissues. Genomic DNA is generally obtained from tumor cells isolated by laser capture microdissection on tissue sections, from tumor cell lines/long-term cultures, and from freshly isolated tumor cells (directly from surgical specimens). Laser capture microdissection has become a powerful technique in isolating tumor cells, especially in acquiring the preneoplastic cells in the precursor lesions. However, it can be labor intensive to obtain a sufficient amount of DNA for assays. Tumor cell lines/long-term cultures, on the other hand, provide a convenient source of pure tumor DNA, but they are not ideal for genomic analyses of human cancer because they may acquire a variety of genetic changes as a result of *in vitro* selection, and those genetic changes may not be relevant to tumorigenesis. Accordingly, it is preferable to use the genomic DNA of purified or enriched tumor cells from surgical specimens if fresh tumor samples are available. Many investigators have used immunosorting in which the magnetic beads are bound to antibodies that react to the tumor-associated antigen on cancer cells. The magnet-isolated tumor cells can be directly used to isolate genomic DNA and RNA or they can be cultured for a short term to further expand the tumor cell population. The epithelial origin of the immunosorted carcinoma cells can be confirmed by staining the purified cells with a cytokeratin antibody or by loss of heterozygosity assay. An example

Figure 2. An example of tumor cell isolation from fresh tissue samples

(**A**) Isolated tumor cells using magnetic beads coated with an Epi-CAM antibody (phase contrast). After magnet sorting, almost all tumor cells are bound to the beads (small dark dots). The isolated tumor cells can be directly used for genomic DNA extraction or they can be short-term cultured to expand the tumor cell population. (**B**) Loss of heterozygosity (LOH) assay. This method is to confirm the purity of immunosorted tumor cells because LOH is unique to tumor but not to normal cells. Both tumor cells (T) and the matched normal cells (N) are analyzed in parallel by PCR using four representative microsatellite markers. Attenuation of intensity in one of the two alleles from the tumor sample (T) was observed in microsatellite markers of chromosomes 5, 8, and 22, indicating a highly enriched tumor cell population. Estimation of the percentage of tumor DNA can be performed by comparing the intensity ratio of the two alleles between tumor and normal samples. The marker in chromosome 1 is not informative because the tumor does not have LOH in the particular locus.



of tumor cell isolation and assessment of its purity is shown in Figure 2.

Combined technical platform for cancer gene discovery

Once amplified or deleted chromosomal regions are identified, the next question is how one can effectively search for the culprit oncogenes and tumor suppressors among an overwhelming number of co-amplified or deleted genes within the same chromosomal region. Because oncogenes within an amplicon almost always overexpress whereas the neighborhood genes may or may not, a rational strategy has been developed by parallel analyses of cancer genome and transcriptome in the same tumors to reveal the profiles of gene expression in the genomically amplified regions. This genome–transcriptome combined approach has allowed several investigators to successfully narrow down the candidate genes [23,42–45]. The other approach expected to significantly facilitate the identification of oncogenes and tumor suppressors is the mutational analysis. Oncogenes and tumor suppressors are characterized not only by DNA copy number changes but also by somatic point mutations because both mechanisms complement each other in tumorigenesis. For example, c-Myc, KRAS, and EGFR are found to be amplified in some tumors and somatically mutated in others of the same tumor type. Similarly, tumor suppressors can be inactivated as a result of homozygous deletion or somatic point mutations. These mechanisms lead to a decrease or a complete abolishment of gene expression. Thus, candidate genes located in the chromosomal regions of interest (identified by digital karyotyping, array CGH and ROMA) can be analyzed for point mutations using a large panel of tumor DNA samples.

Conclusion

The availability of the human genome database creates a new era for biomedical research. New technical platforms that take advantage of the human genome sequences are now available, allowing us to comprehensively analyze complex cancer genomes. The recent development of digital karyotyping, array-based CGH, ROMA, high-throughput mutational analysis, and other emerging techniques provides unprecedented opportunities in discovering new oncogenes, tumor suppressors, and drug-resistant genes, which holds great promise to develop new strategies for diagnosis and treatment of this devastating disease. Further refinement and modification of these techniques will improve their performance including reproducibility, resolution, and throughput in the years to come.

Acknowledgments

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Amplification of a chromatin remodeling gene, Rsf-1/HBXAP, in ovarian carcinoma

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A genomewide technology, digital karyotyping, was used to identify subchromosomal alterations in ovarian cancer. Amplification at 11q13.5 was found in three of seven ovarian carcinomas, and amplicon mapping delineated a 1.8-Mb core of amplification that contained 13 genes. FISH analysis demonstrated amplification of this region in 13.2% of high-grade ovarian carcinomas but not in any of low-grade carcinomas or benign ovarian tumors. Combined genetic and transcriptome analyses showed that Rsf-1 (HBXAPalpha) was the only gene that demonstrated consistent overexpression in all of the tumors harboring the 11q13.5 amplification. Patients with Rsf-1 amplification or overexpression had a significantly shorter overall survival than those without. Overexpression of Rsf-1 gene stimulated cell proliferation and transform nonneoplastic cells by conferring serum-independent and anchorage-independent growth. Furthermore, Rsf-1 gene knockdown inhibited cell growth in OVCAR3 cells, which harbor Rsf-1 amplification. Taken together, these findings indicate an important role of Rsf-1 amplification in ovarian cancer.

digital karyotyping | gene amplification | oncogene

Gene amplification is a common mechanism underlying oncogenic activation in human cancer (1). Amplifications of cyclin E, HER2/neu, AKT2, and L-Myc have been reported in ovarian cancer, and it is expected that many unknown oncogenic amplifications remain to be identified. Recent advances in molecular genetic techniques and the success of the human genome assembly have provided investigators new opportunities to explore cancer genome in great details and to identify novel cancer-associated genes. Digital karyotyping has recently been developed to provide a genomewide analysis of DNA copy number alterations at high resolution (2) and has been applied in cancer genetic studies (3–6). The principle of digital karyotyping is based on extracting and counting the 21-bp sequence tags that represent different loci in human genome. Populations of tags can be directly matched to the unique loci in genome assembly, and digital enumeration of tags provides quantitative measure of DNA copy number along chromosomes. The major advantage of digital karyotyping is that it directly counts the sequence tags, thus providing an unbiased and precise digital readout of DNA copy numbers. The current study has applied this new technology to search for DNA copy number alterations in high-grade ovarian serous carcinoma, the most common and lethal type of ovarian cancer.

Materials and Methods

Tissue Samples. Tissue samples were obtained from the Department of Pathology at The Johns Hopkins Hospital between 1990 and 2004. Effusion (peritoneal and pleural) samples were obtained from the Norwegian Radium Hospital in Norway. All ovarian carcinomas were of serous type from sporadic cases. Acquisition of tissue specimens and clinical information was approved by an institutional review board (The Johns Hopkins University) or by the Regional Ethics Committee (Norway).

Digital Karyotyping. Carcinoma cells were affinity purified by using magnetic beads conjugated with the Epi-CAM antibody (Dynal, Oslo). The purity of tumor cells was confirmed by immunostaining with an anti-cytokeratin antibody, CAM 5.2 (Becton Dickinson, San Jose, CA) and samples with greater than 95% epithelial cells were used in this study. Digital karyotyping library construction and data analysis were performed by following the protocol in refs. 2 and 3. Approximately 120,000 genomic tags were obtained for each digital karyotyping library. After removing the nucleotide repeats in the human genome, the average of filtered tags was 66,000 for each library. We set up a window size of 300 for the analysis in this study. Based on Monte Carlo simulation, the parameters used in this study can reliably detect >0.5-Mb amplicon with >5-fold amplification with >99% sensitivity and 100% positive predictive value.

FISH and Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were arranged onto tissue microarrays to facilitate FISH analysis. Three representative cores (1.5-mm diameter) from each tumor were placed on the tissue microarrays. Bacterial artificial chromosome clones containing the genomic sequences of the 11q13.5 amplicon at 77.05–77.23 Mb (RP11–1107J12) and EMSY at 75.88–76.09 Mb (CTD-2501F13) were purchased from Bacpac Resources (Children's Hospital Oakland, CA) and Invitrogen (Carlsbad, CA), respectively. The RP11–846G12 bacterial artificial chromosome clone, located at 11q11 (55.88–56.05 Mb), was used as the control probe. The method for FISH has been detailed in ref. 3. Two individuals who were not aware of the tumor grade and clinical information evaluated FISH signals. Approximately 100 tumor cells were examined for each specimen. Amplification of the Rsf-1 and EMSY genes was defined as a ratio of the gene probe signal to the control probe signal exceeding 2.

A mouse monoclonal anti-Rsf-1 antibody (gift from Danny Reinberg, University of Medicine and Dentistry of New Jersey, Piscataway, NJ) was used in the immunohistochemistry study. Immunohistochemistry was performed by standard protocol with an EnVision+System peroxidase kit (DAKO, Carpinteria, CA).

Quantitative Real-Time PCR. Real-time PCR for genomic DNA copy numbers and gene expression levels was performed by using methods described in ref. 7, and PCR primers were listed in the Tables 1 and 2, which are published as supporting information on the PNAS web site. PCR reactions were performed by using an iCycler (Bio-Rad, Hercules, CA). For quantitative PCR performed with genomic DNA, we used a cutoff ratio of 2.2 to define genomic amplification. This cutoff value was determined as the mean + 2 standard deviations based on quantitative PCR analyses of normal

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Abbreviations: OSE, ovarian surface epithelial; shRNA, short hairpin RNA; siRNA, short interfering RNA.

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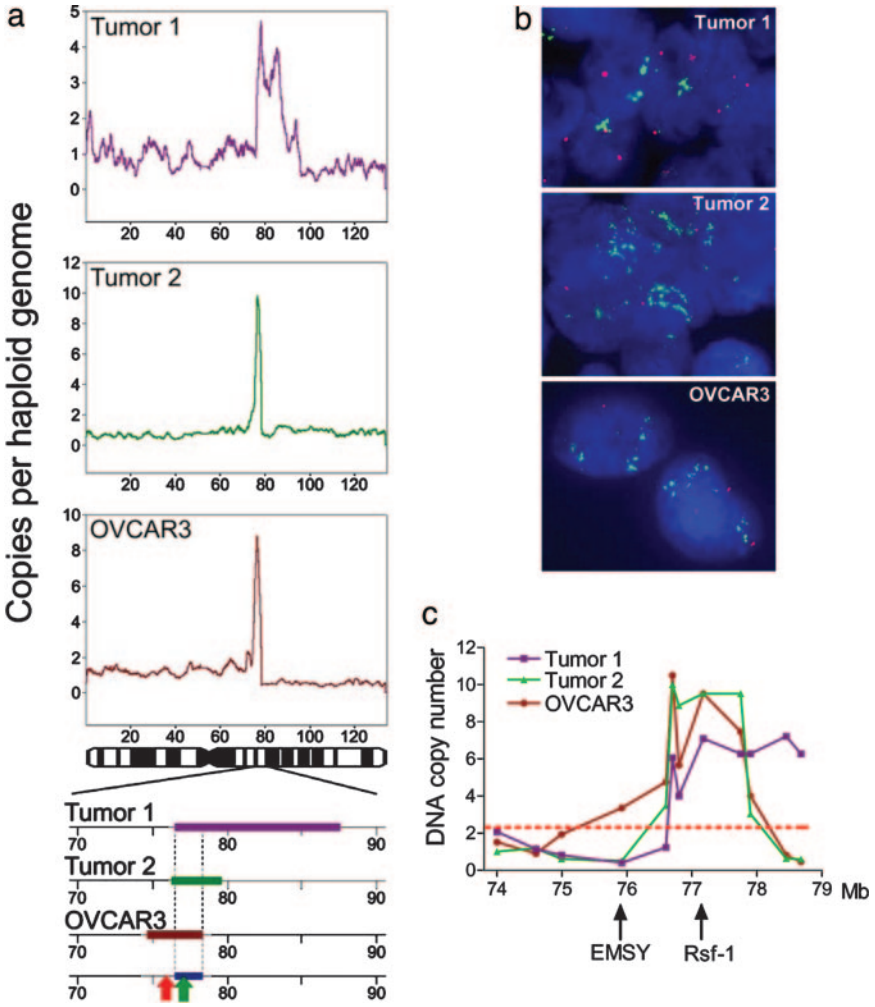

Fig. 1. Identification of the 11q13.5 amplicon in ovarian cancers. (*a*) Digital karyotyping identified three ovarian carcinomas that contained discrete amplifications at 11q13.5 region. Alignment of the amplicons revealed a common region of amplification (blue line in *Bottom*) spanning from 76.6 Mb to 78.4 Mb at chromosome 11q. Red and green arrows indicate the physical locations of EMSY and Rsf-1, respectively. (*b*) Validation of 11q13.5 amplification was performed by FISH analysis in the same three tumors by using a probe (green) located within the minimal amplicon of 11q13.5 and a control probe (red) located at 11q11 (21 Mb centromeric to the minimal amplicon). (*c*) 11q13.5 amplification was further validated in the three tumors by using quantitative real-time PCR on genomic DNA. For each tumor, an increase in the DNA copy number (*y* axis) is present at a specific subchromosomal region that corresponds to the amplification identified by digital karyotyping. The dashed line indicates a cutoff of 2.2 that represents the threshold for amplification with a confidence level of 97.5%.

diploid cells by using all of the primer sets. This cutoff value gave a confidence level of 97.5%.

Cell Proliferation Assay. Cells were seeded in 96-well plates at a density of 4,000 cells per well. The cell number was determined indirectly by the fluorescence intensity of SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR) by using a microplate reader (Fluostar, BMG, Durham, NC). Data were expressed as the mean ± 1 standard deviation from five replicates in each experimental group. Anchorage-independent growth assay was performed as described in ref. 8. Data were expressed as the mean ± 1 standard deviation from triplicates.

Short Interfering RNA (siRNA)-Mediated Knockdown of Rsf-1 Expression. Three siRNAs that targeted Rsf-1 were designed and their sense sequences were as follows: GGAAAGACAUCUCUACUAUUU, UAAAUGAUCUGGACAGUGAUU, and GGACUUACCUUCAACCAAUUU. Control siRNA (off-target control, catalog no. D-001210-02-05) was purchased from Dharmacon (Lafayette, CO). Cells were seeded in 96 wells and transfected with siRNAs by using oligofectamine (Invitrogen). BrdUrd uptake and staining were performed by using a cell proliferation kit (Amersham Pharmacia, Buckinghamshire, U.K.). Apoptotic cells were detected by using an annexin V staining kit (BioVision, Mountain View, CA). The percentage of BrdUrd-positive and annexin V positive cells was determined by counting ≈300 cells from each well in 96-well plates. The data were expressed as mean ± 1 standard deviation from triplicates.

Statistical Method for Clinical Correlation. Overall survival was calculated from the date of the primary surgery for ovarian tumors to the date of death or last followup. Patients with Rsf-1 amplification and without amplification had similar age distributions and received optimal tumor debulking surgery, followed by carboplatin and taxol-based chemotherapy. The data were plotted as Kaplan–Meier curves, and the statistical significance was determined by the Log-rank test. Data were censored when patients were lost to followup. In a Cox proportional hazard model, the *P* values were assessed by using a likelihood ratio test as implemented by the "survival" package in the statistical programming language R (www.r-project.org). Student *t* test was used to examine the statistical significance in the difference of growth assay data.

Results

Digital Karyotyping of Ovarian Carcinomas. Digital karyotyping was used to evaluate the genomic alterations in seven ovarian cancer samples, including six high-grade ovarian serous carcinomas and one ovarian cancer cell line, OVCAR3. Analysis of the genomic tag densities along chromosomes revealed a discrete amplification at chromosome 11q13.5 in three libraries, including two high-grade ovarian carcinomas and the OVCAR3 cell line. No evidence of other amplification cores was detected in chromosome 11 in any of the ovarian cancer libraries (Fig. 6, which is published as supporting information on the PNAS web site). Alignment of these three amplicons delineated an overlapping region of amplification, spanning from 76.6 to 78.4 Mb on the chromosome 11q (Fig. 1*a*). Examination of the RefSeq database in the human genome assem-

bly (July 2003 freeze, University of California, Santa Cruz) revealed that 13 genes were completely located within the minimal amplicon (Fig. 7, which is published as supporting information on the PNAS web site). EMSY gene, which has recently been reported as a candidate oncogene in breast and ovarian carcinomas, was located at 76 Mb (9), close to but outside the minimal region of the amplification (Fig. 1*a*). Two methods were used to validate the digital karyotyping results. First, dual-color FISH was performed to validate the 11q13.5 amplification in these three tumors by using a bacterial artificial chromosomes probe located at the 11q13.5 minimal amplicon (Fig. 7) and a control probe located at 11q11 (21 Mb centromeric to the minimal amplicon). As shown in Fig. 1*b*, we found that all three amplified tumors defined by digital karyotyping showed distinct 11q13.5 amplification. Second, quantitative real-time PCR was performed to measure the DNA copy numbers at 12 loci flanking and within the amplicon, including the EMSY gene in these three tumors (Fig. 1*c*). We found that increases in the DNA copy number were present at subchromosomal regions similar to the amplifications delineated by digital karyotyping. Furthermore, the fold of DNA copy number increase detected by quantitative PCR is at similar levels to that of digital karyotyping. A cutoff ratio of 2.2 was used to search for amplifications with >97.5% confidence, and the delineated common region of amplification (from Loc220032 locus to FLJ23441 locus) was consistent with that derived from digital karyotyping.

To determine the frequency of the 11q13.5 minimal amplicon, we performed dual-color FISH on 211 paraffin-embedded ovarian tissue specimens by using the FISH probe located at the minimal amplicon and the control FISH probe that is the same as described above (located on 11q11). The advantage of selecting the control FISH probe on the same chromosomal arm as the minimal amplicon is that it could facilitate distinguishing chromosome duplication from gene amplification, the latter involving smaller subchromosomal region (10). Using this method, we found 11q13.5 amplification in 16 of 121 (13.2%) high-grade serous carcinomas. In contrast, 11q13.5 gene amplification was not detected in any of 40 low-grade serous carcinomas, 14 serous borderline tumors, 19 benign cystadenomas, and 17 normal ovaries. Thus, 11q13.5 amplification was detected exclusively in high-grade serous carcinomas. Among the 16 tumors with 11q13.5 amplification, 5 cases showed a homogeneous staining region pattern, 3 cases showed a high level gain (>4.5-fold), and the remaining 8 tumors exhibited a moderate gain (between 2.5- and 4-fold). It should be noted that in addition to the 16 tumors with discrete amplification, we observed 11q polysomy in another 14 tumors based on an equal number of signals for both 11q13.5 and control probes. These tumors were not considered to have amplification specific to the 11q13.5 region in this study.

To further elucidate the physical relationship between EMSY and the minimal amplicon, we performed dual-color FISH in the same set of tumor samples by using EMSY probe and same control probe. We found that EMSY was amplified in 12 of 117 (10.3%) high-grade serous carcinomas that were available for analysis. All 12 EMSY amplified carcinomas also demonstrated amplification at 11q13.5 minimal amplicon. Conversely, 4 of the 16 carcinomas that contained 11q13.5 minimal region amplification did not harbor EMSY amplification (Fig. 8, which is published as supporting information on the PNAS web site). This result indicated that 11q13.5 minimal amplicon is more frequently amplified than its neighborhood region that contained EMSY gene in high-grade serous carcinomas.

Transcript Analysis of the 11q13.5 Minimal Amplicon. To identify the potential amplified oncogene within the 11q13.5 amplicon, we applied an approach based on the rationale that a tumor-driving gene, when amplified, almost always overexpresses to activate the tumorigenic pathway, whereas coamplified "passenger" genes that are unrelated to tumor development may or may not do so (10).

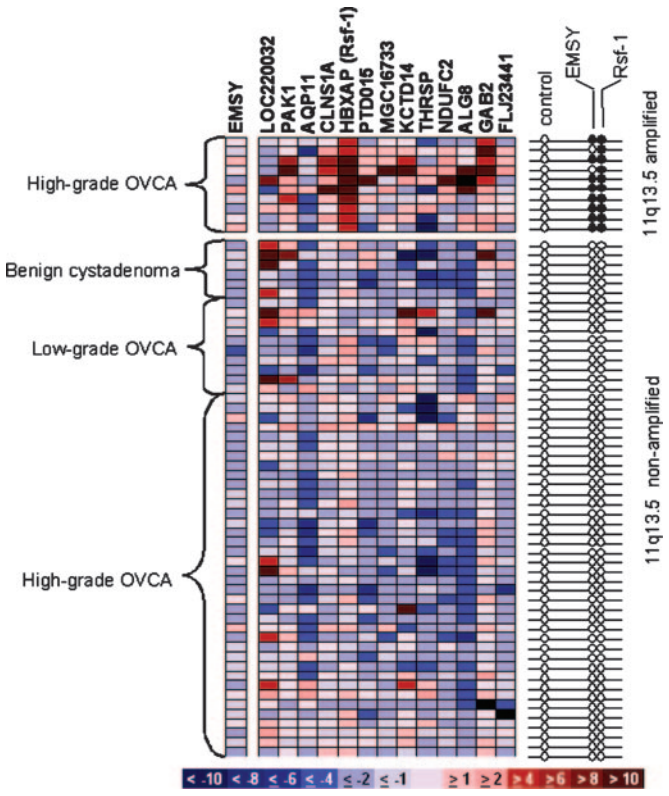

Fig. 2. Gene expression analysis of the 11q13.5 amplicon in ovarian tumors. Quantitative real-time PCR was performed for all 13 genes located within the minimal amplicon in benign cystadenomas, low-grade ovarian carcinomas and high-grade ovarian carcinomas with or without 11q13.5 amplification. The expression level of each gene (left to telomeric) in individual specimen is shown as a pseudocolor gradient based on the relative expression level of a given specimen to the normal ovarian surface epithelium. (*Right*) The amplification status of Rsf-1, EMSY, and the 11q11 (control locus for FISH) for each specimen was determined by FISH analysis. Filled circles indicate amplification, and open circles indicate no amplification.

Therefore, we searched for genes with both DNA amplification and transcript up-regulation in the same tumor samples. Ten high-grade ovarian carcinomas that contained 11q13.5 amplification and had their frozen tissues available were analyzed by quantitative real-time PCR to assess mRNA levels in all of the genes within the minimal amplicon. The same assay was also performed in six benign cystadenomas, 10 serous borderline tumors, and 36 high-grade carcinomas that did not contain 11q13.5 amplification. Freshly brushed ovarian surface epithelium (kind gift from M. J. Birrer, National Cancer Institute, Rockville, MD), which has been considered as an appropriate normal control, was used for normalization of gene expression (11). We used the Wilcoxon test to compute and compare the difference in gene expression levels between 11q13.5 amplified versus nonamplified high-grade carcinomas. We found that among the genes within the minimal amplicon, Rsf-1 (HBXAP) had the most significant difference ($P = 8.5 \times 10^{-6}$) in expression levels between 11q13.5 amplified and nonamplified specimens. Furthermore, Rsf-1 was the only gene demonstrating consistent overexpression among the amplified tumors. Accordingly, Rsf-1 was prioritized for further characterization in this study. EMSY mRNA levels were also measured in parallel. We observed that although the EMSY gene was coamplified in eight of the tested samples, its RNA level was not consistently up-regulated as five of the tumors that harbored EMSY amplification down-regulated EMSY mRNA expression (Fig. 2).

Correlation of Rsf-1 Protein Overexpression and Gene Amplification. To demonstrate a more comprehensive correlation between Rsf-1 gene amplification and protein expression, we performed immu-

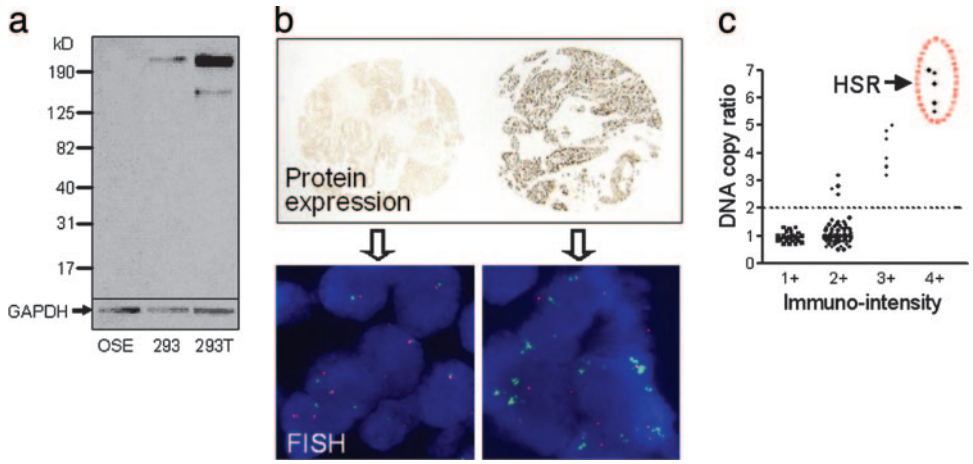

Fig. 3. Correlation of Rsf-1 DNA copy numbers and Rsf-1 protein expression in high-grade ovarian carcinomas. (*a*) The specificity of the anti-Rsf-1 antibody is demonstrated by Western blot analysis. 293, human embryonic kidney (HEK) 293 cells; 293T, HEK293 cells transfected with a full-length Rsf-1 gene. A predominant band of Rsf-1 protein at 215 kDa that represents the full-length Rsf-1 gene is detected in 293T cells. The faint lower band represents the degradation product of Rsf-1 protein. Endogenous Rsf-1 expression is also observed in 293 cells but not in OSE cells. (*Lower*) The GAPDH expression as the loading control. (*b Left*) A high-grade carcinoma shows weak Rsf-1 immunoreactivity (1+) and does not display Rsf-1 gene amplification. (*Right*) A high-grade tumor demonstrates an intense Rsf-1 immunoreactivity (4+) and displays Rsf-1 gene amplification. (*c*) Rsf-1 protein expression correlates with the Rsf-1 gene copy number in high-grade ovarian carcinomas. Tumors with the highest copy number of Rsf-1 DNA [manifested as homogenous staining regions (HSR)] express the highest level of Rsf-1 protein (4+). Tumors that lack Rsf-1 amplification demonstrate weak to moderate Rsf-1 immunoreactivity (1+ and 2+). Each dot represents an individual specimen. Among 16 amplified tumors, there are 15 available for immunohistochemistry.

nohistochemistry with an anti-Rsf-1 monoclonal antibody on the same panel of tissues used in FISH analysis. The specificity of the Rsf-1 monoclonal antibody has been demonstrated in ref. 12 and was independently confirmed in this study (Fig. 3*a*). Overall, there was a statistically significant correlation between Rsf-1 gene amplification and Rsf-1 immunoreactivity ($P < 0.001$, Spearman correlation). We found that 11q13.5 nonamplified tumors demonstrated either weak (1+, 21% of tumors) or moderate (2+, 74% of tumors) Rsf-1 immunoreactivity (Fig. 3 *b* and *c*). In contrast, all of the tumors with Rsf-1 amplification demonstrated an immunointensity of 2+-4+, with the most intense immunoreactivity (4+) found in those with a homogeneous staining region pattern ($n = 5$) and a strong immunoreactivity (3+, $n = 6$) found in those with high-fold DNA gain (3- to 5-fold) (Fig. 3 *b* and *c*). Four tumors with mild gain (2- to 3.5-fold) in the Rsf-1 DNA copy number demonstrated moderate immunointensity (2+), and, thus, they were similar to the majority of the high-grade tumors without Rsf-1 amplification. This finding is likely attributed to the semiquantitative nature inherent to immunohistochemistry in scoring mild to moderate immunointensity because such limitation in scoring Her2/neu immunointensity has been reported in ref. 13.

Clinical Significance of 11q13.5 Amplification and Rsf-1 Overexpression. Amplification of the Rsf-1 locus and Rsf-1 overexpression were correlated with clinical outcome in patients with high-grade ovarian serous carcinoma. Because the FISH probe used to assess 11q13.5 amplification contained the whole Rsf-1 coding region (Fig. 7), it allowed us to use the same FISH data and analyze the clinical significance of Rsf-1 amplification. A total of 107 of 121 patients were available for survival analysis. The other 14 tumors harboring chromosome11q polysomy were excluded in the analysis because polysomy was considered as duplication of chromosomal arms or large genomic segments and could not simply be grouped to either Rsf-1 amplified or nonamplified cases.

We found that all 107 patients had advanced stage high-grade serous carcinomas (the majority at FIGO stage III). Among them, 16 patients who had Rsf-1 amplification in their tumors had a shorter overall survival compared with those without amplification ($P = 0.015$; Log rank test) (Fig. 4*a*). The median overall survival was 29 months [95% confidence index (CI): 18.8–39.1 months] for the amplified group and 36 months (95% CI: 24.3–47.7 months) for the nonamplified group. Quantitative real-time PCR was also used to measure Rsf-1 mRNA in tumor cell pellets from 53 effusion samples that were not feasible for FISH analysis. An arbitrary cutoff of the expression level (>2.4 fold compared with normal ovarian surface epithelium) was used to assign specimens to either high expression ($n = 11$) or low expression ($n = 42$) groups. The results indicated that high levels of Rsf-1 mRNA expression (>2.4 fold) were correlated with poor outcome ($P = 0.037$; Log rank test) (Fig. 4*b*) with median overall survival of 19 months (95% CI: 14.5–23.6) in patients with Rsf-1 mRNA overexpression and 38 months (95% CI: 28.3–47.8) in patients without Rsf-1 mRNA overexpression. Rsf-1 amplification and overexpression appeared as independent prognostic factors based on a multivariate analysis adjusted for patient age, clinical stage, and differentiation status of tumor histology.

To further test whether the clinical significance of Rsf-1 amplification and overexpression depended on the arbitrary cutoffs, we performed a survival analysis by using continuous variables in a Cox proportional hazard model. The *P* values assessed by a likelihood

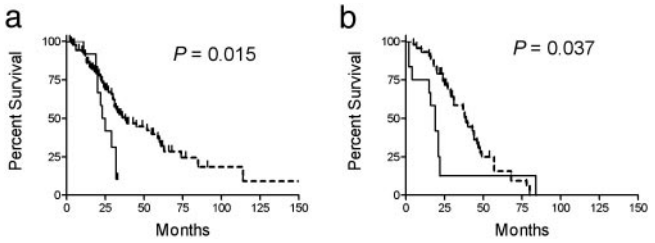

Fig. 4. Rsf-1 amplification and overexpression correlate with shorter overall survival in patients. (*a*) Kaplan–Meier survival analysis shows that Rsf-1 amplification (solid line, $n = 16$) is associated with a shorter overall survival compared with tumors without Rsf-1 amplification (dashed line, $n = 91$) ($P = 0.015$, Log-rank test). (*b*) Quantitative real-time PCR in effusion samples of ovarian high-grade serous carcinomas demonstrates that Rsf-1 overexpression (> 2.4 fold of normal ovarian surface epithelium; solid line; $n = 11$) correlates significantly with shorter overall survival than those with a low expression level (< 2.4 fold; dash line; $n = 42$) ($P = 0.037$, Log-rank test).

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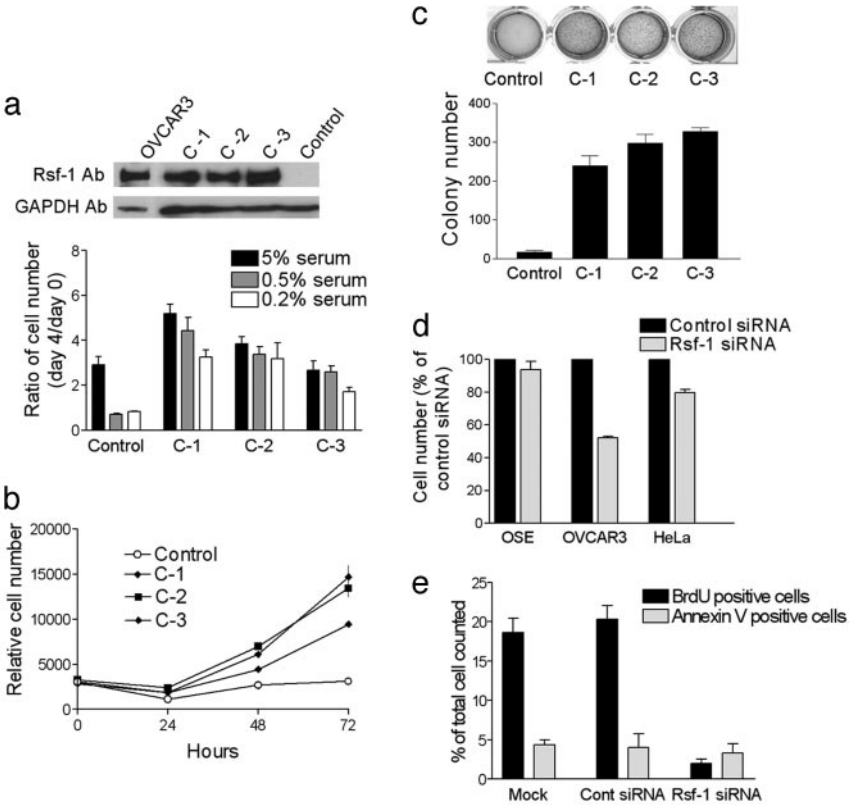

Fig. 5. Functional analyses of Rsf-1 expression. (*a*) Western blot analysis shows that Rsf-1-transfected RK3E clones (C-1, -2, and -3) express Rsf-1 protein with a predominant molecular mass of 215 kDa that is similar to the endogenous Rsf-1 protein expressed in OVCAR3 cells. Control RK3E cells, which are transfected with an empty vector, do not express Rsf-1 protein. As compared with the control RK3E cells, Rsf-1 clones continue proliferating at low serum concentrations (0.5% and 0.2%). (*b*) The Rsf-1 clones demonstrate a higher proliferative activity than the vector only control, as evidenced by a time-dependent increase in cell number at low (0.5%) serum-containing medium. (*c*) Anchorage-independent assay demonstrates that colonies observed in the Rsf-1 clones are more than those in the vector only control. (*d*) Effects of Rsf-1 gene knockdown. Knockdown of Rsf-1 significantly reduces cell number in OVCAR3 cells that harbor Rsf-1 amplification and in HeLa cells that express Rsf-1. In contrast, Rsf-1 siRNA has only a minimal effect on cell growth in OSE cells that do not express detectable Rsf-1. (*e*) Rsf-1 targeting siRNA reduces cell proliferation as measured by the percentage of BrdUrd-positive cells but not in control siRNA or in nontreated (mock control) OVCAR3 cells. The percentage of apoptotic cells as measured by annexin V staining is similar among Rsf-1 siRNA, control siRNA, and nontreated OVCAR3 cells.

ratio test were 0.025 for FISH assay and 0.0013 for real-time PCR. These results further indicated that Rsf-1 amplification and overexpression were significantly correlated with poor survival, independent of the cutoffs.

In this study, we further compared the statistical significance in correlating gene amplification and overexpression with overall survival of Rsf-1 gene to those of EMSY gene. For gene amplification, Rsf-1 had a more significant P value than that of EMSY (0.015 vs. 0.08). Similarly, for gene expression, Rsf-1 expression also had a more significant P value than EMSY expression by using a low stringent cutoff value of >1-fold (0.037 vs. 0.153).

Functional Analyses of Rsf-1 Expression. We therefore stably expressed the Rsf-1 gene in the nonneoplastic epithelial cells, RK3E, to assess whether Rsf-1 expression induced transformation (*Supporting Methods*, which is published as supporting information on the PNAS web site). RK3E cells have been used to evaluate the oncogenic potential of GL1, c-Myc, and mutant β-catenin and were considered as an appropriate *in vitro* model for oncogenic transformation (14–16). Using quantitative real-time PCR, we found that ovarian serous carcinomas predominantly expressed the full-length form of the Rsf-1 gene (or HBXAPa), therefore RK3E cells were transfected with a vector expressing the full-length Rsf-1, and three independent clones were randomly selected for functional analyses. Western blot analysis confirmed the Rsf-1 expression in these clones (Fig. 5*a*). All of the Rsf-1-expressing clones proliferated better at very low (0.2% and 0.5%) serum concentrations and showed a higher proliferative activity than control RK3E cells (transfected with vector alone) based on increased cell numbers (Fig. 5 *a* and *b*) and BrdUrd incorporation (data not shown). Rsf-1-expressing clones grew anchorage independently as more colonies were observed in Rsf-1-expressing cells than in control cells (Fig. 5*c*). All of the above differences were of statistical significance ($P < 0.001$, t test).

To further determine whether Rsf-1 expression was essential for cell survival in cell lines that overexpress Rsf-1, we used RNA interference to knock down Rsf-1 expression in three cell lines, including OVCAR3 cells (with Rsf-1 amplification and overexpression), HeLa cells (without amplification but with Rsf-1 expression), and ovarian surface epithelial (OSE; without Rsf-1 amplification or expression) cells. The effect of Rsf-1 siRNA in suppressing Rsf-1 expression was confirmed by quantitative real-time PCR (Fig. 9, which is published as supporting information on the PNAS web site). Reduction of Rsf-1 expression significantly inhibited cell growth in Rsf-1-expressing cells, including OVCAR3 and HeLa cells (Fig. 5*d*, $P < 0.001$, t test), with a more prominent inhibitory effect in Rsf-1 amplified OVCAR3 cells. In contrast, the same treatment did not affect cell growth in OSE cells, which had minimal Rsf-1 expression ($P = 0.26$, t test). The inhibition of cell growth after repressing Rsf-1 expression in OVCAR3 was likely a result of growth suppression as the percentage of BrdUrd-labeled cells was significantly decreased in Rsf-1 siRNA-treated cells as compared with control siRNA-treated OVCAR3 cells (Fig. 5*e*, $P < 0.001$). In contrast, the percentage of apoptotic cells as measured by annexin V staining was similar between the Rsf-1 siRNA and control groups. To extend the findings of Rsf-1 gene knockdown *in vitro*, we transfected OVCAR3 cells with Rsf-1 short hairpin RNA (shRNA) before injecting the cells into nude mice (*Supporting Methods*). Western blot analysis demonstrated that Rsf-1 expression was substantially reduced in Rsf-1 shRNA-transfected OVCAR3 cells as compared with the control shRNA-transfected cells (Fig. 10, which is published as supporting information on the PNAS web site). All mice injected with Rsf-1 shRNA-treated OVCAR3 cells develop much smaller intraabdominal xenograft tumors than the mice carrying control (scramble) shRNA-transfected cells (Fig. 10, $P < 0.001$, $n = 5$).

Discussion

This study provides cogent evidence that amplification of Rsf-1 within the 11q13.5 minimal amplicon is involved in ovarian

tumorigenesis based on a comprehensive study including molecular genetics, transcriptome analysis, clinical correlation, and functional characterization. Chromosome 11q13.5 amplification is one of the most frequently amplified regions in human tumors including ovarian, breast, head, and neck carcinomas. The frequency of 11q13.5 amplification in ovarian carcinoma detected in this study (13.2%) is similar to but slightly lower than that previously reported (17%) (9). This result is likely due to more stringent criteria used for FISH analysis in the current study. For example, we have used a reference probe on chromosome 11q arm instead of on chromosome 11 centromere to exclude cases that belong to polysomy or large segment duplication. It should be noted that EMSY was located near the minimal amplicon delineated in the current study. EMSY functions as a BRAC2-interacting gene and was previously thought of as a candidate oncogene for ovarian cancers (9). However, the oncogenic property of EMSY in ovarian tumor was not demonstrated in that study. Furthermore, our findings with a larger scale of ovarian tumor samples did not demonstrate a significant correlation of EMSY gene amplification and mRNA overexpression, a finding arguing against EMSY as the "driver" gene within the amplicon.

Based on our combined genetic and expression analyses, we have found that Rsf-1 is consistently overexpressed in all of the amplified tumors examined. In addition to Rsf-1, several other genes close to Rsf-1, including CLNS1A, ALG8, and GAB2, were coup-regulated in a subset of tumors with 11q13.5 amplification. It would be interesting in the future to determine whether cooverexpression of these genes would further provide growth advantages in the development of ovarian cancer. The association of Rsf-1 amplification/overexpression with worse survival in ovarian cancer patient is similar to oncogenes, including HER2/neu in breast cancer (17, 18) and N-myc in neuroblastoma (19), in which overexpression of both oncogenes stimulates cell proliferation and confers a shorter survival. The mechanism of how Rsf-1 amplification contributes to shorter survival is not known; however, because the mortality of ovarian cancer patients is directly related to the recurrent disease after chemotherapy, it is conceivable that Rsf-1 amplification may confer drug resistance and/or enhance cell proliferation in the chemoresistant recurrent tumors.

Our study with gene overexpression and RNA interference knockdown has established an important functional role of Rsf-1 in ovarian cancer. How can Rsf-1 contribute to tumor progression at the molecular level? Recent *in vitro* studies have indicated that Rsf-1 plays a role in chromatin remodeling (12) and transcriptional regulation (20, 21) that may contribute to tumorigenesis. Rsf-1 has been shown to function as a histone chaperone, whereas its binding partner, hSNF2H, possesses nucleosome-dependent ATPase activity. The Rsf-1/hSNF2H complex (or RSF complex) participates in chromatin remodeling by mobilizing nucleosomes in response to a variety of growth modifying signals and environmental cues. Such nucleosome remodeling is essential for transcriptional activation or repression (22), DNA replication (23), and cell cycle progression (24). Recently, a growing body of evidences has accumulated to support a novel role of chromatin remodeling in cancer (25, 26). For example, mutations and deletions of a hSNF2H homolog, Brg1, were found in different tumor types (27), and furthermore, heterozygous deletion of Brg1 in mice resulted in a cancer-prone phenotype (28, 29). It is plausible that Rsf-1 gene amplification and overexpression in tumor cells could disrupt the homeostatic kinetics in the chromatin remodeling machinery and fine tune gene regulation that facilitates tumorigenesis. Because the current study identifies and characterizes the previously undescribed Rsf-1 gene amplification in ovarian cancer, further studies are required to elucidate the etiological roles of Rsf-1 amplification and overexpression in chromatin remodeling and cancer development.

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